Chapter 3

Interaction of Mimetic Analogs of Insect Kinin Neuropeptides with Arthropod Receptors

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Abstract

Insect kinin neuropeptides share a common C-terminal pentapeptide sequence Phe[1]-Xaa$_1$[2]-Xaa$_2$[3]-Trp[4]-Gly[5]-NH$_2$ (Xaa$_1$[2] = His, Asn, Phe, Ser or Tyr; Xaa$_2$[3] = Pro, Ser or Ala) and have been isolated from a number of insects, including species of Dictyoptera, Orthoptera and Lepidoptera. They have been associated with the regulation of such diverse processes as hindgut contraction, diuresis and the release of digestive enzymes. In this chapter, the chemical, conformational and stereochemical aspects of the activity of the insect kinins with expressed receptors and/or biological assays are reviewed. With this information, biostable analogs are designed that protect peptidase-susceptible sites in the insect kinin sequence and demonstrate significant retention of activity on both receptor and biological assays. The identification of the most critical residue of the insect kinins for receptor interaction is used to select a scaffold for a recombinant library that leads to identification of a nonpeptide mimetic analog. C-terminal aldehyde insect kinin analogs modify the activity of the insect kinins leading to inhibition of weight gain and mortality in corn earworm larvae and selective inhibition of diuresis in the housefly. Strategies for the modification of insect neuropeptide structures for the enhancement of the topical and oral bioavailability of insect neuropeptides and the promotion of time-release from the cuticle and/or foregut are reviewed. Promising mimetic analog leads for the development of selective agents capable of disrupting insect kinin regulated processes are identified that may provide interesting tools for arthropod endocrinologists and new pest insect management strategies in the future.

Introduction

Insect neuropeptides of the insect kinin class share a common C-terminal pentapeptide sequence Phe[1]-Xaa$_1$[2]-Xaa$_2$[3]-Trp[4]-Gly[5]-NH$_2$ (Xaa$_1$[2] = His, Asn, Phe, Ser or Tyr; Xaa$_2$[3] = Pro, Ser or Ala). They have been isolated from a number of insects, including species of Dictyoptera, Orthoptera and Lepidoptera. The first members of this insect neuropeptide family were isolated on the basis of their ability to stimulate contractions of the isolated cockroach hindgut,[1-3] but they are also potent diuretic peptides that stimulate the secretion of primary urine by Malpighian tubules, organs involved in the regulation of salt and water balance.[3-5] In addition, the insect kinins have been implicated in the regulation of digestive enzyme release.[6-8] More recently, insect kinins and/or analogs, have been reported to inhibit weight gain by larvae of the tobacco budworm (*Heliothis virescens*) and corn earworm (*Helicoverpa zea*),[3,9] both serious agricultural pests.

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Neuropeptide Systems as Targets for Parasite and Pest Control, edited by Timothy G. Geary and Aaron G. Maule.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2010		**00-00-2010 to 00-00-2010**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Interaction of Mimetic Analogs of Insect Kinin Neuropeptides with Arthropod Receptors	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Department of Agriculture/ Areawide Pest Management Research Unit,Southern Plains Agricultural Research Center,2881 F/B Road,College Station,TX,77845**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Insect kinin neuropeptides share a common C‑terminal pentapeptide sequence Phe1‑Xaa12‑Xaa23‑Trp4‑Gly5‑NH2 (Xaa12 = His, Asn, Phe, Ser or Tyr; Xaa23 = Pro, Ser or Ala) and have been isolated from a number of insects, including species of Dictyoptera, Orthoptera and Lepidoptera. They have been associated with the regulation of such diverse processes as hindgut contraction, diuresis and the release of digestive enzymes. In this chapter, the chemical, conformational and stereochemical aspects of the activity of the insect kinins with expressed receptors and/or biological assays are reviewed. With this information, biostable analogs are designed that protect peptidase‑susceptible sites in the insect kinin sequence and demonstrate significant retention of activity on both receptor and biological assays. The identification of the most critical residue of the insect kinins for receptor interaction is used to select a scaffold for a recombinant library that leads to identification of a nonpeptide mimetic analog. C‑terminal aldehyde insect kinin analogs modify the activity of the insect kinins leading to inhibition of weight gain and mortality in corn earworm larvae and selective inhibition of diuresis in the housefly. Strategies for the modification of insect neuropeptide structures for the enhancement of the topical and oral bioavailability of insect neuropeptides and the promotion of time‑release from the cuticle and/or foregut are reviewed. Promising mimetic analog leads for the development of selective agents capable of disrupting insect kinin regulated processes are identified that may provide interesting tools for arthropod endocrinologists and new pest insect management strategies in the future.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**22**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

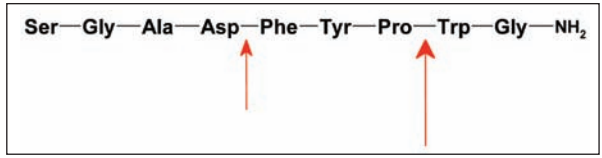

Figure 1. Primary (large arrow) and secondary (small arrow) hydrolysis cleavage sites of tissue-bound peptidases for the natural cricket insect kinin SGADFYPWG-NH_2 (AK-I). From Zubrzak et al (2007).[49]

Unfortunately, insect kinin peptides are unsuitable as research tools for insect neuroendocrinologists and/or pest control agents due to susceptibility to both exo- and endopeptidases in the hemolymph and gut of the insect. These insect neuropeptides are also unsuitably designed for efficient bioavailability, whether delivered via a topical and/or oral route. Members of the insect kinin family are hydrolyzed and therefore inactivated, by tissue-bound peptidases of insects. Two susceptible hydrolysis sites in insect kinins[3] have been reported. The primary site is between the Pro^3 and the Trp^4 residues, with a secondary site N-terminal to the Phe^1 residue in natural extended insect kinin sequences (Fig. 1). Experiments demonstrate that angiotensin converting enzyme (ACE) from the housefly can cleave at the primary hydrolysis site, whereas neprilysin (NEP) can cleave insect kinins at both the primary and secondary hydrolysis sites.[3,10-13] In this chapter, we review what is known about chemical, conformational and stereochemical aspects of the interaction of insect kinins with their receptors and how this knowledge can be harnessed to design and develop biostable mimetic analogs that retain an ability to bind and potentially activate, insect kinin receptors. Strategies for the modification of insect neuropeptides to enhance bioavailability characteristics and the potential to control pest insect populations with mimetic insect kinin analogs that disrupt water and ion balance and/or digestive processes are also discussed.

Functional Analysis of Arthropod Receptors Selective for Insect Kinins

G protein-coupled receptors (GPCR) constitute the largest super family of receptors.[14] GPCR are integral plasma membrane proteins which are characterized by seven hydrophobic transmembrane spanning α-helices, three intracellular loops, three extracellular loops, an amino terminal outside the cell and a carboxy terminal inside the cell.[15,16] On recognizing extracellular primary peptide messengers, GPCR mediate intracellular communication across the cell membrane by coupling to intracellular heterotrimeric G-proteins that activate secondary-messenger pathways. The activated G-protein modulates various intracellular processes that regulate cell function.[17] The ability to selectively regulate GPCR and their signaling pathways may lead to the discovery of novel insecticidal targets.[18]

Holmes et al (2000)[19] used polymerase chain reaction (PCR) and molecular techniques to obtain a cDNA of a novel leucokinin-like peptide receptor, the first known neuropeptide receptor from the Southern cattle tick, *Boophilus microplus* (Acari: Ixodidae). For this, degenerate primers were designed based on the sequence similarity of transmembrane (TM) regions III and VI lymnokinin and vertebrate neurokinin receptors.[19,20] Until the cloning of tick leucokinin-like peptide receptor, this receptor subfamily comprised only one receptor, the lymnokinin receptor, cloned from a mollusc, the pond snail *Lymnaea stagnalis.*[20] The amino acid sequence of the *B. microplus* receptor showed most similarity to the CG10626 *Drosophila melanogaster* gene product and to the lymnokinin receptor from *L. stagnalis,* which has been characterized as a leucokinin-like peptide receptor.[19] The C-terminal end of TM II (residues 103-110) and the first extracellular loop (residues 111-123) are highly conserved among three leucokinin-like peptide receptors the *B. microplus*, *L. stagnalis* and *D. melanogaster* but not in other neuropeptide receptor subfamilies.[19] Similarly, the first eight residues of the C terminus (337-344) are highly conserved among the leucokinin-like peptide receptors and less conserved among the other neuropeptide receptors.[19] The presence of the mRNA in all the life stages of the tick and diverse tissues reflects the importance of this receptor for various functions and suggests that it can be

used as a target for the development of novel and specific acaricides.[19] The receptor from the Southern cattle tick, (*B. microplus*) was stably and functionally expressed in CHO-K1 cells and responded to physiological concentrations of insect kinin peptides as determined by single cell intracellular calcium measurements using confocal fluorescence microscopy.[21] To date no endogenous tick kinin has been identified. Based on the similarity to the tick receptor sequence, Holmes et al predicted the CG10626 *Drosophila* gene product encoded the first known insect leucokinin receptor,[19] which was later confirmed by Radford et al (2002).[22] In the genome of *Drosophila melanogaster*, only one drosokinin receptor is present and only one peptide, drosokinin, is the ligand for this receptor.[22] In the mosquito *Aedes aegypti*, three kinins have been isolated and the single cDNA encoding the three peptides has been cloned.[23,24] The *A. aegypti* kinin receptor is the first cloned from mosquitoes.[25] In *A. aegypti* females the function of insect kinins had been previously investigated in the Malpighian tubule, where they increase fluid secretion and depolarize the transepithelial voltage,[23,24] therefore, cDNA was synthesized from these tissues to isolate the receptor cDNA. The strategy consisted in designing degenerate primers corresponding to transmembrane regions VI and VII of the invertebrate receptors known, the drosokinin, lymnokinin and *Boophilus microplus* myokinin receptors to obtain a specific 280 bp PCR product, followed by 5′ and 3′ RACE to obtain the complete open reading frame sequence. The *Aedes* cDNA sequence was used to improve the prediction of the mosquito *Anopheles gambiae* kinin receptor sequence (ex XP309852; EAA05450 discontinued record) which lacked the first transmembrane region and residues at the C-terminus.[25] The *A. gambiae* receptor symbol is now GPRLKK; the protein Ensembl peptide ID is AGAP010851-PA; and the transcript (ID AGAP010851-RA, see *Anopheles gambiae* genome in www.vectorbase.org) sequence is identical to that predicted by Pietrantonio et al (2005).[25] The *A. aegypti* cDNA encodes a 584 amino acid residue protein of predicted molecular mass of 65.2 kDa.

The mosquito kinin receptor cDNA was expressed in CHO-K1 cells for functional analysis by intracellular calcium measurements. In addition to analysis by confocal fluorescence cytometry mentioned above, the receptor-expressing cells were analyzed by calcium bioluminescence in a plate assay using the calcium reporter aequorin.[25] These analyses showed the mosquito kinin receptor is indeed a multiligand receptor triggering intracellular calcium release in response to the three Aedes kinins and kinin analogs, similarly to the *Boophilus* kinin receptor. The two methodologies showed that the decreasing rank order of potency on the *Aedes* receptor is Aedes kinin 3 followed by Aedes kinin 2 and Aedes kinin 1. These three neuropeptides are significantly different in their EC_{50}. In *Drosophila,* in addition to its localization and enrichment in Malpighian tubules,[26] the receptor is present in the nervous system, hindgut and in gonads of males and females as shown by western blot and immunohistochemistry.[22] In females of *Aedes aegypti,* the receptor transcript was confirmed by PCR in the hindgut and head and in the ovaries by in situ hybridization (Pietrantonio and Jagge, unpublished results). It is clear that in these dipterans, the kinin receptor has additional functions in addition to the initially discovered role in chloride transport in the Malpighian tubule.[28,29] The kinin receptor in the central nervous system has more recently been implicated in pre-ecdysis behavior in *Drosophila melanogaster* and *Manduca sexta.*[30,31] Analysis of the first coleopteran genome of the beetle *Tribolium castaneum* revealed this species is devoid of the kinin receptor.[32] Thus, it appears different orders of insects have evolved diverse (and perhaps equivalent) signaling pathways at least for specific molecular events in diuresis and metamorphosis, functions in which insect kinins are involved in the Diptera.

Chemical, Conformational and Stereochemical Aspects of Receptor Interaction

Myotropic and diuretic assays of tissues in vitro, as well as assays using stably expressed insect kinin receptors, show that the full biological activity of the insect kinins resides in the C-terminal pentapeptide, which is the active core.[5,33,34] Exceptions are the housefly Malpighian tubule fluid secretion assay[35] and the plate assay for an expressed insect kinin receptor from the mosquito *Aedes aegypti*, where the C-terminal pentapeptide core is less potent by several orders of magnitude. Diuretic, myotropic and/or receptor-interaction activity in these assays is completely lost when the C-terminal amide of

the insect kinins is replaced with a negatively charged acid moiety.[36,37] Within the core pentapeptide, the aromatic residues Phe[1] and Trp[4] are the most important for activity whereas a wide range of variability is generally tolerated at position 2, from acidic to basic residues and from hydrophilic to hydrophobic.[5,33] The expressed insect kinin receptor from the mosquito *Aedes aegypti* represents a particular exception, as it clearly prefers an aromatic residue in position 2, which is consistent with the presence of aromatic residues in position 2 of all three of the native *Aedes* kinins.[37]

Despite the steric bulk in the backbone of the Aib-containing insect kinin analog FF(Aib)WGa, it nevertheless elicits a very strong calcium bioluminescence response in both tick and mosquito receptors.[37] This is in agreement with the potent activities of Aib containing analogs observed in a cricket Malphigian tubule fluid secretion assay, an in vivo housefly diuretic assay and a cockroach hindgut myotropic assay.[3,10] In the mosquito receptor, it is statistically equipotent with the positive-control, agonist analog FFFSWGa; whereas in the tick receptor it is an order of magnitude more potent than this same agonist. Therefore, it is the most potent peptide analog yet reported for the tick receptor. The steric bulk of the Aib residue also restricts the number of conformations available to the backbone of this analog and provides some insight into the conformation adopted by the insect kinins at the two receptors. A previous solution conformation study using both NMR spectroscopic data and molecular dynamics calculations concludes that the analog adopts only two major turn conformations. These consist of a turn over residues Phe[1] through Trp[4], comprising 60% of the population and another over residues Phe[2] through Gly[5], comprising the remaining 40%.[11,38,39] Similarly, NMR spectroscopic data and molecular dynamics calculations on an active head-to-tail, cyclic analog (*cyclo*(AFFPWG)) reveal the presence of two major turn types within the active core region of the insect kinins, analogous with those observed in the Aib-containing analog. The more rigid of the two conformations featured a *cis*Pro in the third position of a Type-VI β-turn over core residues 1-4, or Phe-Phe-**Pro**-Trp. ROESY spectra supported a well-defined Cβ-*exo*/Cγ-*endo* pucker for the *cis*Pro ring that was observed in unrestrained molecular dynamics for this cyclic analog. The other less rigid turn system involved a *trans*Pro and encompassed residues 2-5, or Phe-**Pro**-Trp-Gly. From unrestrained molecular dynamics calculations, the most favorable *cis*Pro structure had an intramolecular energy about 7 kcal/mole lower than the most favorable *trans*Pro structure, consistent with NMR data that indicated that the *cis*Pro 1-4 turn structure was the predominant conformation in solution by a 60:40 ratio.[11,39,40] This is in agreement with systematic studies on linear peptides with Pro[3] in which the flanking aromatic residues promote the formation of Type VI β-turns in aqueous solution. Such turns are further enhanced when small, hydrophyllic residues (i.e., Asp, Ser, Thr, **Gly** or Asn) follow the aromatic-Pro-aromatic motif,[77] as occurs in the cyclic insect kinin analog. The molecular modeling studies further indicate that interactions between the aromatic sidechains in positions 1 and 4 help to stabilize the turn over residues 1-4 containing the *cis*Pro configuration, which might otherwise be expected to be less energetically favorable than *trans*Pro.[11,39]

In an effort to provide definitive evidence that the most populous *cis*Pro Type VI β-turn over residues Phe[1] through Trp[4] represented the 'active conformation' for receptor interaction, insect kinin analogs incorporating restricted conformation components that preferentially mimic a *cis* peptide bond and a Type VI β-turn were synthesized and evaluated. NMR studies with insect kinin analogs incorporating either the tetrazole or 4-aminopyroglutamate (APy) (Fig. 2), moieties

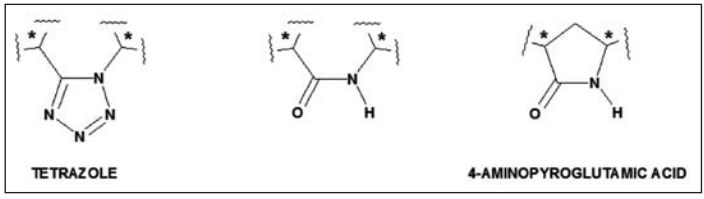

Figure 2. A comparison of the structures of the tetrazole (ψ(CN_4), left) and 4-aminopyroglutamic acid (APy; right) motifs, mimics of the *cis*-peptide bond (middle) and a Type VI β-turn. From Nachman et al (2004).[41]

Table 1. Estimated potencies (EC_{50}) and percentage of maximal bioluminescence response of different analogs in reference to FFSWGa tested on tick (BmLK3) receptor expressing cell lines.[44] EC_{50} are an estimate of the concentration required to induce a half-maximal response. The tick receptor response is compared with activity of the APy analogs[41,42] and tetrazole analogs[40,42] in a cricket diuretic assay.

	Tick Receptor, (BmLK3 Cell Line)		Cricket Diuretic Assay	
Analogs	**EC_{50} (μM)**	***Maximal Response (%)**	**Stimulation of Malpighian Tubule Fluid Secretion EC_{50} (10^{-8} M)**	**Maximal Response (%)**
Amino Pyroglutamate analogs				
1518, Ac-RF(APy)WGa (R,S)	1.6	65	0.7	93
RN2, Ac-RF(APy)WGa (S,S)	11.1	48	14	93
RN3, Ac-RF(APy)WGa (S,R)	N.D.	0	12	96
RN4, Ac-RF(APy)WGa (R,R)	N.D.	45	7	83
Tetrazole analogs				
FF_{ψ}**(CN_4)**(dA)WGa (L,D)	N.D.	0	(43)$^{\psi}$	
FF_{ψ}**(CN_4)**AWGa (L,L)	N.D.	32	34	100
$F(dF)_{\psi}$**(CN_4)**AWGa (D,L)	N.D.	47	2.2$^{\Phi}$	94$^{\Phi}$
$F(dF)_{\psi}$**(CN_4)**(dA)WGa (D,D)	N.D.	0	58	100
Positive control				
FFSWGa	0.57	100	-	-

*Maximal response is the maximal bioluminescence response of each analog expressed as a percentage of the maximal response of the active core agonist FFSWGa (positive control) at 10 μM. N.D.: The analog was tested but was either not very active or was not active at lower molarities; thus an EC_{50} could not be determined. $^{\psi}$The analog demonstrates partial antagonism of the native achetakinins, limiting the response to 50% of the maximum. $^{\Phi}$Nachman et al, unpublished data.

that mimic one turn over the other, indicate a predominant population of a β-turn involving the Phe^1 to Trp^4 region.[40,41] These restricted conformation analogs in the natural L,L (or S,S) configuration demonstrate significant retention of both diuretic activity in the cricket Malpighian tubule fluid secretion assay and interact with the expressed insect kinin receptor from the tick *Boophilus microplus* (Table 1).

A more detailed investigation of the stereochemical requirements for the interaction of the restricted-conformation analogs provided evidence for optimal turn-region stereochemistry in both the cricket Malpighian tubule fluid secretion assay and the expressed insect kinin receptor from the tick *Boophilus microplus*. For this investigation, all four stereochemical variants of tetrazole (L,L; L,D; D,L; and D,D) and APy (*2S,4S*; *2R,4S*; *2S,4R*; and *2R,4R*) moieties were incorporated into the C-terminal region of the insect kinin sequence (see Table 1). In the in vitro cricket diuretic assay, the most active agonists were the tetrazole analog FFψ**(CN_4)**AWGa (D,L) (Nachman and Coast, unpublished data) and APy analog Ac-RF(APy)WGa (*R,S*) with EC_{50}'s of 22 and 7 nM, respectively.[42] The optimal stereochemistry for the two turn mimic moieties in the cricket diuretic assay could therefore be identified as (D,L) and (2R,4S), respectively. The other three stereochemical analog variants (*2S,4S*)-, (*2S,4R*)- and (*2R,4R*)-APy retain agonist activity, but were less potent than the (*2R,4S*)

variant by at least an order of magnitude. Conformational studies in aqueous solution indicate that the (*2R,4S*)-APy analog is considerably more flexible than the other three variants, which may explain its greater potency.[42] The (L,L)- and (D,D)-tetrazole stereochemical variants retain agonist activity, but were less potent than the (D,L) variant by at least an order of magnitude; whereas, in contrast, the (L,D)-tetrazole analog demonstrated an ability to antagonize the diuretic response of natural achetakinins.[40] It was suggested that the change in stereochemistry of the α-carbon at the N-terminal end of the tetrazole moiety from L to D appears to inhibit the activation response by interfering with the electrostatic interaction that occurs between the side chains of the Phe^1 and Trp^4 that allows these two critical side chains to present an optimal aromatic surface to the receptor.[41]

As with the cricket diuretic assay, an evaluation of the restricted conformation analogs in the expressed tick receptor demonstrated that the APy moiety was a superior mimic of the active conformation of the insect kinins over the tetrazole. Three of the four APy analogs demonstrated significant agonist activity. The (*2R,4S*)- and (*2S,4S*)-APy analogs demonstrated EC_{50} values of 1.6 μM in the bioluminescence plate assay (Maximal response: 65%) and 11 μM (Maximal response: 48%), respectively.[43] Analog (*2R,4R*)-APy was not active enough to determine an EC_{50} value and reached a maximal response of 45% only at concentrations of 10 μM and higher; whereas the (*2S,4R*)-APy analog proved essentially inactive. Only two of the tetrazole analogs demonstrated any appreciable activity; (D,L) (Maximal response: 47%) and (L,L) (Maximal response: 32%) and neither were active enough to allow the calculation of an EC_{50} value. Unlike the cricket diuretic assay, both the (D,D)- and (L,D)-tetrazole analogs were essentially inactive on the tick receptor; and no antagonist activity was observed for the (L,D) analog. Nonetheless, the optimal stereochemistry for activation of the tick receptor proved to be the same as for agonist activity in the cricket diuretic assay—(*2R,4S*) for the APy moiety and (D,L) for the tetrazole moiety.[43]

It should be noted that the NOVOstar bioluminescence method used to evaluate the response of the insect kinin analogs on expressed receptors reported in these studies is less sensitive as compared with a less practical confocal fluorescence cytometry method previously employed.[21] The bioluminescence plate assay is between 50 to 70-fold less sensitive. This difference should be taken into account when estimating the potency that these analogs would likely demonstrate in in vitro or in vivo physiological bioassays. For example, an analog that is active at 1 μM in the bioluminescence assay would be active at 20 nM or lower in bioassays.[44]

The critical nature of the sidechains of Phe^1 and Trp^4 and the *cis*Pro, Type VI turn conformation to the activity of the insect kinins was also confirmed by evaluation of a small series of pseudotetrapeptide analogs that featured only these minimal constructs. These 'minimalist' analogs, based on an amino piperidinone carboxylate scaffold, retained very weak, but statistically significant diuretic activity in the in vitro cricket Malpighian tubule secretion bioassay.[45]

In summary, structure/conformation-activity data suggest that a receptor interaction model for the insect kinins can be proposed in which the C-terminal pentapeptide region adopts a Type VI turn over residues Phe^1 to Trp^4 and that the aromatic side chains of Phe^1 and Trp^4 are oriented towards the same region and interact with the receptor (Fig. 3). Conversely, the side chain of residue 2 lies on the opposite face pointing away from the receptor surface, which explains why this position is more tolerant to changes[46] (Fig. 3).

Interaction of Biostable Insect Kinin Analogs with Receptors and Activity in In Vitro and In Vivo Bioassays

Members of the insect kinin family are hydrolyzed and therefore inactivated, by tissue-bound peptidases of insects. Two susceptible hydrolysis sites in insect kinins[3] have been reported. The primary site is between the Pro^3 and the Trp^4 residues, with a secondary site N-terminal to the Phe^1 residue in natural extended insect kinin sequences (Fig. 1). Experiments demonstrate that angiotensin converting enzyme (ACE) from the housefly can cleave at the primary hydrolysis site, whereas neprilysin (NEP) can cleave insect kinins at both the primary and secondary hydrolysis sites.[3,10-13]

Incorporation of β-amino acids can enhance both resistance to peptidase attack and biological activity[47,48] and this strategy has not been previously applied to insect neuropeptides. Recent work has described the synthesis of a number of analogs of the insect kinin C-terminal pentapeptide core

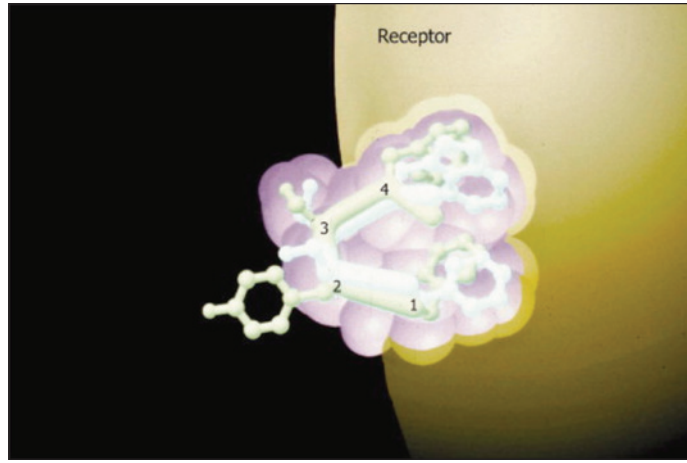

Figure 3. The currently accepted receptor-interaction model of an insect kinin C-terminal pentapeptide core region in a 1-4 turn, the active conformation. The side chain aromatic groups of Phe[1] and Trp[4] of the core region are both important for an agonist response. The indole group of Trp[4] is the most critical for receptor binding. From Nachman et al (2002).[40]

in which the critical residues Phe[1], Pro[3] and Trp[4] and or adjacent residues, are replaced with β^3-amino acid and/or their β^2-amino acid counterparts. β^3-amino acids feature an additional methylene group incorporated between the alpha carbon and the carboxyl group of the natural α-amino acid structure, whereas in β^2-amino acids the additional methylene group is placed between the alpha carbon and the amino group.[47,48]

Several noteworthy β^3-amino acid insect kinin analogs are listed in Table 2. The examples in Table 2 represent the replacement of only noncritical α-amino acids with β-amino acids. In the single-replacement analog 1460 (Ac-RFF(**β^3Pro**)WGa), a β^3-amino acid is placed adjacent to the primary peptidase-susceptible peptide bond (between Pro[3]-Trp[4]) of the insect kinins.[3] In the two double-replacement analogs, β-amino acids are placed in proximity to both primary and secondary peptidase-susceptible sites (Fig. 3). It was anticipated that these β-amino acids would afford some measure of resistance to hydrolysis by the peptidases that degrade and therefore inactivate, the natural peptides. Indeed, analogs 1460, 1577 (Ac-R(**β^3Phe**)FF(**β^3Pro**)WGa) and 1578 (Ac-RF(**β^3Phe**)-(**β^3Pro**)WGa) exhibit significantly enhanced resistance to peptidases that attack at the susceptible sites[49] (Table 2). Double-replacement analog 1578 demonstrates the greatest

Table 2. β-amino acid-containing insect kinin analogs—enhancement of peptidase resistance[49]

		% Hydrolysis	
Peptide Analog		**Human NEP**	***Drosophila* ACE**
LK I	DPAFNSWGa	99.8	81.6
1460	Ac-RFF(**β^3Pro**)WGa	15.4	10.7
1577	Ac-R(**β^3Phe**)FF(**β^3Pro**)WGa	0	3.5
1578	Ac-RF(**β^3Phe**)-(**β^3Pro**)WGa	2	16.5

Table 3. Estimated potencies (EC_{50}) and percentage of maximal bioluminescence response of different analogs in reference to FFFSWGa and the minimum active core sequence FFSWGa, tested on mosquito (E10) and tick (BmLK3) receptor expressing cell lines.[44,49] EC_{50} are an estimate of the concentration required to induce a half-maximal response.

	Mosquito Receptor (E10 Cell Line)		Tick Receptor (BmLK3 Cell Line)		Cricket Malpighian Tubule Fluid Secretion	
Analogs	**EC_{50} (10^{-8}M)**	***Maximal Response (%)**	**EC_{50} (10^{-8}M)**	***Maximal Response (%)**	**EC_{50} (10^{-10}M)**	***Maximal Response (%)**
Single Beta Analogs						
1460 Ac-RFF(β³**Pro**)WGa	36.7	50	28.7	72	0.3	100
1656 Ac-RFFP(β²**Trp**)Ga	N.D.	0	68.5	57	400	97
Double Beta Analogs						
1577 Ac-R(β³**Phe**)FF (β³**Pro**)WGa	65.3	65	49.5	64	1	100
1578 Ac-RF(β³**Phe**-β³**Pro**)WGa	N.D.	7	68.7	62	10	100
Positive Controls						
FFFSWGa	68.5	100	27.0	100	-	-
FFSWGa Insect kinin active core	N.D.	29	59.0	100	-	-
AK-III DQGFNSWGa	-	-	-	-	3	100

*Maximal response is the maximal bioluminescence response of each analog expressed as a percentage of the maximal response of the known agonist FFFSWGa (positive control) at 10 μM. for tick and mosquito receptors; or to achetakinin-III (AK-III) in the cricket diuretic assay. N.D.: The analog was tested but was either not sufficiently active to determine an EC_{50} value.

resistance to the peptidases ACE and NEP among those listed in Table 2. Under conditions in which the natural insect kinin LK-I is degraded 100% by NEP and 82% by ACE, analog 1578 shows no degradation by NEP and only 4% by ACE. These three analogs are also blocked at the N-terminus with an acetyl (Ac) group, which confers resistance to hydrolytic degradation by an additional class of peptidases, the aminopeptidases.[50]

Despite the incorporation of an additional methylene group (-CH_2-) within the backbone of the C-terminal pentapeptide core region, the single replacement of amino acids with their β-amino acid counterparts led to significant retention of activity in expressed kinin receptors from the tick *Boophilus microplus* and mosquito *Aedes aegypti*, very evolutionarily distant arthropod species. The single-replacement analog 1460, that involves modification of the Pro^3, was the most potent of the β-amino acid analogs in both mosquito and tick receptors[44] (Table 3). Analog 1460 proved to be more active than the positive control agonist FFFSWGa in the mosquito and considerably more active than the minimum active core sequence FFSWGa. In the tick, 1460 was equipotent with the positive control agonist FFFSWGa and more active than the C-terminal pentapeptide active core. Analog 1460 therefore is a nonselective, biostable agonist for these two receptor systems. However, it is clear that the mosquito receptor is considerably more sensitive to modifications at Trp^4 than the tick receptor. In particular, the mosquito receptor is intolerant to the replacement of Trp^4 with β²Trp, as 1656 (Ac-RFFP(β²**Trp**)Ga) is inactive[44] (Table 3). This complete loss of activity by the β²Trp analog 1656 in the mosquito receptor cell line may result from an increase in

the distance between the α-carbons of the critical aromatic residues at positions 2 and 4 over what is found in the natural peptide. The tick receptor remains relatively tolerant to the introduction of a methylene group between the α-carbon and the amino group of Trp^4, as the potency of 1656 was not statistically different from the C-terminal pentapeptide core analog FFSWGa and the analog retained 57% of the maximal response.[44] By virtue of this difference in receptor-interaction requirements, analog 1656 is a selective agonist for the tick receptor. Although not as yet tested, analog 1656 would be expected to exhibit significantly enhanced peptidase resistance as well.

Double replacement analog 1577 features modification of noncritical regions; the noncritical Pro_3 residue and the residue just outside of the critical C-terminal pentapeptide core region. Accordingly, this analog retains significant activity in both the mosquito and tick receptors, demonstrating potency that exceeds that of the minimum active core and is not significantly different from that of the positive control agonist FFFSWGa.[44] It is a nonselective, biostable agonist of both arthropod receptors.

Double replacement analog 1578 features modification of noncritical residues Phe^2 and Pro^3 that effectively changes the distance between the sidechains of the critical residues Phe^1 and Trp^4. Like analog 1656, this modification is tolerated to a much greater extent by the tick receptor than the mosquito receptor. Consequently, the analog 1578 demonstrates retention of activity in the tick receptor assay, with a potency that is not significantly different from that of the active core analog FFSWGa; but remains essentially inactive in the mosquito receptor system[44] (Table 3). Thus, biostable agonist analog 1578 demonstrates selectivity between the two expressed arthropod receptor cell lines.

The β-amino acid insect kinin analogs have not as yet been evaluated in in vitro mosquito or tick diuretic assays. However, data for these analogs has been reported in an in vitro insect kinin Malpighian tubule fluid secretion assay from the cricket *Acheta domesticus*[49] and are listed in Table 3 as well. As with the two expressed arthropod receptors for the insect kinins, analog 1460 was the most potent β-amino acid analog in the cricket fluid secretion bioassay, demonstrating a potent EC_{50} of 0.3×10^{-10}M and a 100% maximal response. This analog exceeds by an order of magnitude the activity of at least one of the native achetakinins, AK-III ($EC_{50} = 3 \times 10^{-10}$M) and essentially matches the activity of the most potent of achetakinins. Although inactive in the mosquito insect kinin receptor, the analog containing β^2Trp (1656) retains activity ($EC_{50} = 40 \times 10^{-10}$M; 97% maximal response) in the in vitro cricket diuretic assay, although considerably reduced from the native achetakinins.[49] This analog demonstrates a degree of selectivity for the expressed tick receptor over receptors of the mosquito and cricket. As with the two expressed arthropod insect kinin receptors, the double-replacement analog 1577 retained potent activity in the cricket diuretic assay, with an EC_{50} value of 1×10^{-10}M and a100% maximal response[49] (Table 3). While virtually inactive in the expressed mosquito receptor, the double-replacement analog 1578 retained activity in the cricket fluid secretion assay with an EC_{50} of 10×10^{-10}M and a 100% maximal response. The assay results obtained with the β-amino acid insect kinin analogs suggest that the receptor associated with the Malpighian tubules of the cricket is more similar to the expressed insect kinin receptor from the tick than that of the mosquito.

Another set of insect kinin analogs that demonstrate enhanced resistance to peptidases feature a replacement of the third position of the C-terminal pentapeptide core (Pro or Ser) with α-aminoisobutyric acid (Aib), a sterically hindered α,α-disubstituted amino acid which effectively protects the primary tissue-bound peptidase hydrolysis site.[3,10] Incorporation of a second Aib residue adjacent to the secondary peptidase hydrolysis site, as with analog **(Aib)**FS**(Aib)**WGa (analog 781), further enhances the biostability.[3] For instance, over a one hour period a natural helicokinin is completely degraded by either peptidases bound to Malpighian tubule tissue from the moth *Helicoverpa zea* or a pure preparation of the peptidase NEP. Conversely, the double-Aib analog **(Aib)**FS**(Aib)**WGa remains to the extent of 100% and 90%, respectively, over a three to four hour period.[3,10] In the cricket Malpighian tubule assay, analog 781 exceeds the activity of the native achetakinins by an order of magnitude. Analogous Aib-containing insect kinin analogs also

demonstrate activity that either approaches or exceeds that of the positive control FFFSWGa in expressed receptors for mosquito *Aedes aegypti* and the tick *Boophilus microplus*, respectively.[78]

Due to the different structure-activity requirements of the insect kinin receptor found with the Malpighian tubule fluid secretion assay in the housefly (*Musca domestica*), the in vitro activity of analog 781 is over four orders of magnitude less than that of the native muscakinin. Nonetheless, the in vivo diuretic activity of 781 is *equipotent* with that of the native muscakinin. Evidence indicates that analog 781 demonstrates a longer hemolymph residence time in the housefly than the peptidase-susceptible muscakinin and it is this extended presence that likely explains the remarkable in vivo activity observed for this analog.[3] Therefore, biostable characteristics can enhance the in vivo activity of insect kinin analogs.

While in vivo activity studies of biostable insect kinin analogs have not as yet been completed for the cricket, tick or mosquito, some in vivo results have been obtained in larvae of the corn earworm moth, *Helicoverpa zea*.[3] Injection of helicokinins into developing larvae of the related moth *Heliothis virescens* has been observed to inhibit weight gain.[9] A helicokinin-II analog (VRFSSWGa) and a biostable Aib helicokinin analog **pQ**RFS**(Aib)**WGa (Hek-Aib) were injected daily (0.5 nmoles) into 5-day old *H. zea* larvae for 5 or 6 days until pupation occurred. The helicokinin analog demonstrated a developmental trend that reached a peak on day 5 posttreatment, with a statistically insignificant 20% weight reduction as compared with controls. In contrast, the biostable, helicokinin Aib analog elicited a stronger, statistically significant effect spanning days 4-7, reaching a peak at day 5 of about a 50% reduction in mean larval weight as compared with controls. The time of pupation was delayed by a factor of 25%.[3]

Seinsche et al (2000)[9] demonstrated that the weight gain inhibition they observed in *H. virescens* is accompanied by an increase in the excretion of water in the feces, consistent with the diuretic activity previously observed in crickets,[27] flies,[10,51] as well as the lepidopteran *H. virescens*.[9] However, the higher excretion of fluid alone could not sufficiently explain the observed loss of weight in insect kinin-treated tobacco budworm larvae. The authors further speculated that the insect kinins could have induced a starvation signal in the *Heliothis* larvae, resulting in mobilization of energy stores and a decreased efficiency in exploiting digested nutrients.[9] For instance,[52] demonstrated that the levels of insect kinin-like immunoreactivity in the haemolymph increased 10-fold in crickets starved for 48 h without access to water. They speculated that the insect kinins may play a role in energy mobilization during starvation. Support for this idea comes from work by[53] who found that nanomolar doses of insect kinins led to increases in lipid concentration similar to that caused by the adipokinetic hormone and also inhibited protein synthesis. To this should be added the likely contribution made by the reported ability of insect kinins to inhibit release of protease and amylase digestive enzymes from the lepidopteran midgut,[7,8] which would have prevented the diet from being efficiently digested. Together with the increased excretion of fluid and induction of a starvation response, an inhibition of digestive enzyme release may have led to the weight losses observed in both *H. virescens*[9] and *H. zea*[10] treated with insect kinins and/or analogs.

C-Terminal Aldehyde Analogs of Insect Kinins

Aldehydes can form reversible imine bonds with amino groups. Peptide analogs containing reversible binding moieties at the C-terminus, such as an aldehyde, have been reported to inhibit various classes of proteolytic enzymes.[54-56] It has been further postulated that a C-terminal aldehyde moiety could form a covalent, reversible Schiff base (imide linkage) with the amino group of a Lys residue[57] in an insect kinin receptor pocket, thereby modifying the ligand-receptor interaction characteristics of the resulting insect kinin analog. Enhanced and/or modified receptor-interaction characteristics could lead to an enhancement and/or modification of in vivo activity. Evaluations of two C-terminal aldehyde kinin analogs, R-LK-CHO (Fmoc-RFFPWG-H) and V-LK-CHO (Boc-VFFPWG-H), in developmental and diuretic assays have been reported.[34,58] Both aldehyde analogs demonstrated in vitro stimulation of fluid secretion in isolated cricket Malpighian tubules in the physiological concentration range and full efficacy, thereby providing evidence that they could interact with an insect kinin receptor site.

R-LK-CHO (EC_{50} = 250 nM) was approximately 10 fold less active than V-LK-CHO (EC_{50} = 30 nM) in this cricket diuretic assay, but both were several orders of magnitude less active than their parent hexapeptide insect kinin analogs.[34] Regardless of the presence of an aldehyde or an amide group at the C-terminus, the V-containing analogs were 10-fold more potent than the R-containing analogs in this in vitro diuretic assay system.

H. Zea *Larval Weight Gain Inhibition Bioassay*

Injection of R-LK-CHO into 5-day old *H. zea* larvae induced statistically significant reductions in weight gain in comparison with control animals on days 2 and 4-6 at the 5 nm dose, but not at 500 pm. Day 6 larvae experienced a significant reduction in weight gain at the 5 nm dose, with treated animals observed to be about 65% of the weight of controls. No significant difference in mortality was observed between treated and control groups. Thus, the incorporation of the aldehyde group in the C-terminus did appear to enhance the activity of the insect kinins in this in vivo weight gain inhibition assay.[34] A previous study had determined that the observed inhibition of weight gain in *H. zea* larvae on days 3-6 post treatment with the *normal* insect kinin VRFSSWGamide was not statistically significant. Significant weight gain was not observed in this previous study until a peptidase-resistant, Aib-containing insect kinin analog (pQRFS(Aib)WG-amide) was synthesized and evaluated in the in vivo larval assay. Corn earworm larvae treated with this fortified analog were observed to be about 50-60% of the weight of control animals,[3] with no significant increase in mortality.

The other aldehyde analog, V-LK-CHO, demonstrated a more pronounced effect than R-LK-CHO in the *H. zea* larval weight gain inhibition assay. V-LK-CHO induced significant reductions in weight gain on days 2 and 4 through 6, after initiation of the treatment at *both* the 500 pm and 5 nm dose. At day 6, treated larvae were observed to be 65% and 40% that of the weight of the control animals at doses of 500 pm and 5 nm, respectively (Fig. 4). In order to reduce weight gain to 65% of that of control animals, treatments of 500 pm for V-LK-CHO and 5 nm of R-LK-CHO were required, a 10-fold difference in potency (Fig. 4). Notably, in those animals treated with V-LK-CHO a significant increase in mortality was observed at both doses (45%{500 pm}and 67%{5 nm})[34] (Fig. 4).

The significant increase in mortality observed in larvae treated with V-LK-CHO is not likely a result of some general toxic effect of the aldehyde moiety itself, as increased mortality was not observed in R-LK-CHO, which also features a C-terminal aldehyde.[34] However, the enhanced reductions in larval weight gain induced by the insect kinin C-terminal aldehyde analogs are consistent with a modified interaction of these analogs with the insect kinin receptor over that occurring with the normal C-terminal amide peptides.

In Vitro and In Vivo Housefly Diuretic Bioassays

As mentioned previously, insect kinin analogs in which the C-terminal amide was replaced by an aldehyde moiety retained an ability to stimulate fluid secretion by cricket Malpighian tubules. Although a reliable in vivo assay for diuresis in crickets did not exist, such an assay was available for houseflies.[51,59] Thus, the insect kinin aldehydes V-LK-CHO, R-LK-CHO and analogs were evaluated for activity on housefly Malpighian tubules (Table 4).

Neither of the two aldehyde analogs V-LK-CHO and R-LK-CHO stimulated fluid secretion, although notably tubules exposed to R-LK-CHO did not respond when subsequently challenged with a supramaximal concentration (10 nM) of native muscakinin (Musdo-K). In contrast, the same concentration of Musdo-K elicited a marked diuretic response in tubules that had first been exposed to V-LK-CHO. The inhibitory effect of R-LK-CHO on the diuretic activity of 10 nM Musdo-K was dose-dependent with an IC_{50} of 12 μM (Fig. 5), which compares favorably with the EC_{50} of an N-terminal truncated Musdo-K analog of similar length.[35,58]

Other aldehyde analogs were also tested on housefly tubules for diuretic activity and their ability to inhibit stimulation of fluid secretion by 10 nM Musdo-K. Of these, Fmoc-RAHPWG-H, which closely resembles R-LK-CHO, had diuretic activity with an EC_{50} of 2.3 μM. On the other hand, an Aib containing aldehyde analog, Fmoc-RFF(**Aib**)WG-H, had no effect on fluid secretion

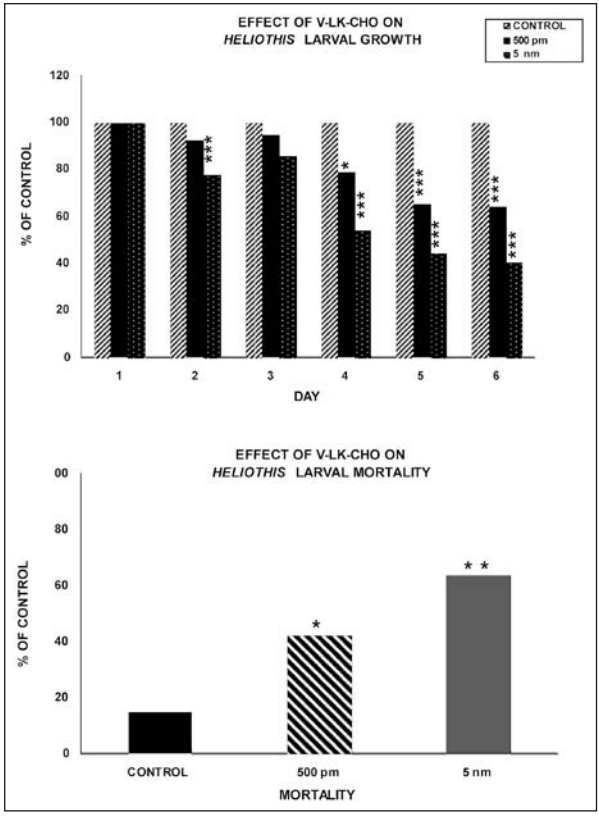

Figure 4. The graph at the top illustrates the effects of daily injections of 500 pmoles of the insect kinin C-terminal aldehyde analog V-LK-CHO (Boc-VFFPWG-H) on inhibition of weight gain in 5-day old larvae of the corn earworm *H. zea* expressed as a percentage of saline injected control animals. Each test group contained at least 10 animals and the experiment was repeated five times (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; indicates the difference from the control group is statistically significant). The graph at bottom illustrates the percent mortality observed in these *H. zea* larvae injected with either 500 pm or 5 nm of V-LK-CHO for 5 days compared with saline injected controls. From Nachman et al (2003).[34]

Table 4. In vitro diuretic activity of C-terminal insect kinin analogs in the housefly Musca domestica[34]

Insect Kinin Analog	Stimulation of Malpighian Tubule Fluid Secretion– EC_{50} (10^{-6}M) (% Maximal Response)
Boc-Val-Phe-Phe-Pro-Trp-Gly-H (V-LK-CHO)	Inactive
Fmoc-Arg-Phe-Phe-Pro-Trp-Gly-H (R-LK-CHO)	(12*) (Inhibitory)
Fmoc-Arg-**Ala-His**-Pro-Trp-Gly-H	2.3 (100)
Fmoc-Arg-Phe-Phe-**Aib**-Trp-Gly-H	Inactive

*IC_{50} for inhibitory activity

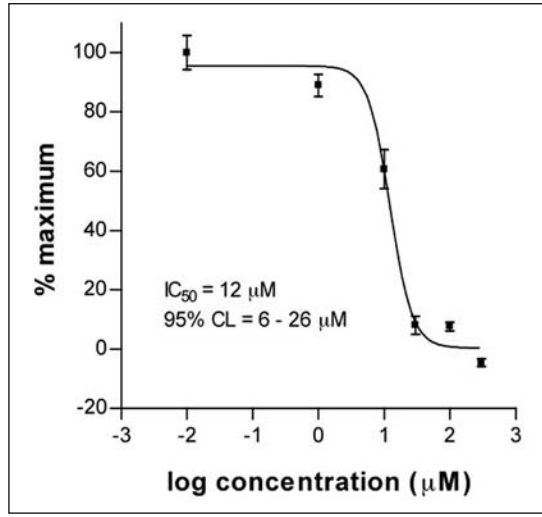

Figure 5. Dose-response curve for R-LK-CHO showing the percentage inhibition of the diuretic activity of 10 nM Musdo-K. Data points are the means of 5-7 determinations and vertical lines ±1 S.E.M. From Nachman et al (2007).[58]

and, in common with V-LK-CHO, it did not inhibit the diuretic activity of 10 nM Musdo-K.[58] The inhibitory activity of R-LK-CHO is thus highly specific and not a generalized effect of the aldehyde moiety.

The ability of R-LK-CHO to prevent stimulation of fluid secretion by Musdo-K was of considerable interest, because the analog might be useful as a probe to investigate the role of the native kinin in the control of diuresis in vivo. By using a high sensitivity flow through humidity analyzer the episodic excretion of urine from intact houseflies was recorded.[59] R-LK-CHO inhibits the in vivo activity of Musdo-K as evidenced by a marked reduction in the amount of urine voided over the initial 45min post-injection period in flies injected with 50 pmol each of the analog and the kinin compared with the kinin alone. R-LK-CHO was also shown to reduce the volume of urine voided during a diuresis initiated by the injection of 3 μL saline alone, although a larger dose (250 pmol) of the aldehyde was needed for a pronounced effect, probably because of the expanded hemolymph volume[58] (Fig. 6). The diuretic response to hypervolemia is partly attributable to the release of Musdo-K from neurohaemal sites into the circulation and the inhibitory effect is consistent with a selective effect of R-LK-CHO at the kinin receptor.

Injection of 1 μL distilled water is a more effective stimulant of diuresis than is the injection of 3 μL of saline, with total urine loss over 3h being more than double that in saline injected flies. This difference in part reflects an autonomous response of the Malpighian tubules, which secrete at higher rates when the osmotic concentration of the bathing fluid is reduced, as would occur after injecting flies with distilled water.[51] R-LK-CHO would not necessarily be expected to have any effect on the autonomous response of Malpighian tubules to haemolymph dilution and yet it reduced the total amount of urine voided over 3h from flies injected with 1 μL of distilled water by almost 50% (Fig. 7).

The markedly reduced urine output from flies injected with 1 μL distilled water containing 50 pmol R-LK-CHO suggests this analog has a toxic effect on Malpighian tubules and in support of this we have show that it blocks stimulation of fluid secretion by thapsigargin, a SERCA inhibitor and by ionomycin, a calcium ionophore.[58] Kinin neuropeptides use Ca^{2+} as a second messenger to open a paracellular or transcellular chloride conductance pathway. This is mimicked by thapsigargin and ionomycin, which increase the level of intracellular calcium by promoting Ca^{2+} release for intracellular stores and the influx of Ca^{2+} from the bathing fluid, respectively. The ability of

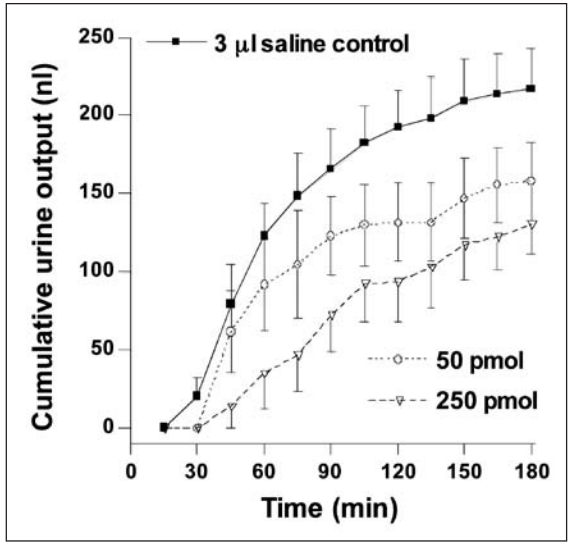

Figure 6. R-LK-CHO attenuates the in vivo diuretic response to hypervolemia induced by the injection of 3 µL of saline. The volume of urine excreted by individual flies was measured over 15 min periods for 3 h in insects injected with saline alone (solid squares, solid line) or saline containing either 50 pmol (open circles, dotted line) or 250 pmol (open triangle, dashed line) of the aldehyde analog. Data points show the means ± 1 S.E.M of the cumulative urine output in 10 (saline alone) and 6 (+ R-LK-CHO) flies. From Nachman et al (2007).[58]

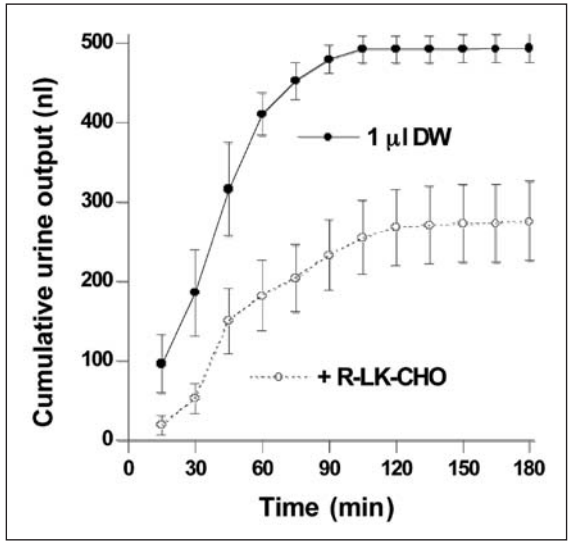

Figure 7. R-LK-CHO significantly reduces the amount of urine excreted by flies injected with 1 µL of distilled water. The volume of urine excreted by individual flies was measured over 15 min periods for 3 h in insects injected with distilled water alone (solid squares, solid line) or with distilled water containing 50 pmol (open circles, dotted line) of the aldehyde analog. Data points show the means ± 1 S.E.M of the cumulative urine output in 7 (distilled water) and 9 (+ R-LK-CHO) flies. From Nachman et al (2007).[58]

R-LK-CHO to block the activity of these pharmacological probes shows it cannot be an antagonist of the kinin receptor, but must act downstream of the second messenger pathway.

At present, the cellular action(s) of R-LK-CHO on Malpighian tubules that leads to prevention of stimulation of fluid secretion by Musdo-K, thapsigargin and ionomycin is unknown. It is clearly not a generalized toxic effect, because the same analog has diuretic activity in the cricket Malpighian tubule assay. Moreover, closely related aldehyde analogs tested on housefly tubules either have no activity (V-LK-CHO and Fmoc-RFF**(Aib)**WG-H) or stimulate fluid secretion (Fmoc-R**A**HPWG-H). R-LK-CHO may act as a 'magic bullet' and bind with the kinin receptor on housefly tubules, become internalized and thereby gain access to intracellular processes that couple a rise in intracellular calcium levels to the opening of the chloride conductance pathway.[58]

Compounds in the hemolymph, including pesticide toxins, are actively transported into the lumen of the Malpighian tubules and their rate of elimination is dependent on the rate of fluid secretion.[60,61] At high rates of excretion, the toxins do not reach the high concentrations that would allow them to diffuse back into the hemolymph down a concentration gradient. An agent capable of selective depression of fluid secretion would be expected to allow pesticides to achieve higher concentrations in the hemolymph; and, in turn, likely reduce the amount of toxin required to kill an insect. Whatever the mode of action, the selective activity of R-LK-CHO on housefly tubules represents an important milestone and lead in the long-term goal of the development of environmentally-friendly insect management agents based on the insect kinins.

Nonpeptide Mimetic Agonists/Antagonists of Expressed Insect Kinin Receptors

Perhaps the ultimate goal in the search for biostable, bioavailable analogs would be the design and/or discovery of nonpeptide mimetic agonists or antagonists of the insect kinins. The availability of expressed insect kinin receptors can accelerate the discovery process through the evaluation of nonpeptide libraries. A recent biorational approach has based the selection of a nonpeptide library on the presence, within its structure, of the side chain moiety of the most critical residue of the peptide (Nachman, unpublished). As discussed in an earlier section, the most critical residues for the interaction of the insect kinins with expressed receptors from the tick *Boophilus microplus* and mosquito *Aedes aegypti* have been determined to be the Phe[1] and Trp[4] within the C-terminal pentapeptide core region.[11,37,39] Data obtained from an in vitro Malpighian tubule fluid secretion assay indicate that a C-terminal pentapeptide insect kinin analog in which the Phe is replaced with an Ala demonstrates an antagonist response against native achetakinins, whereas the analog in which Trp is replaced with Ala is devoid of activity.[62] Furthermore, a C-terminal aldehyde analog in which Ala replaces Phe retains weak activity in an in vitro cricket diuretic assay.[58] This would suggest that Trp, which contains an indole side chain moiety, represents the most critical amino acid for the binding of insect kinins with the receptor. Consequently, a 400 member nonpeptide library based on the imidopyrido**indole** ('Ipi') scaffold[63] was constructed (Fig. 8) and evaluated in expressed insect kinin receptors from the tick *Boophilus microplus* and mosquito *Aedes aegypti*. One of the Ipi

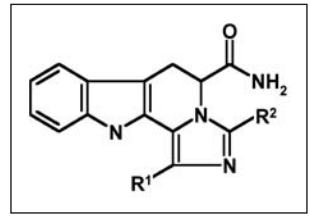

Figure 8. Structure of a rationally-designed, recombinant nonpeptide library based on an imidopyridoindole ('Ipi') scaffold. The scaffold contains an indole group (highlighted with a circle), which appears in the side chain of Trp, the most critical residue within the insect kinin active core region for receptor interaction.

recombinant library analogs demonstrated significant activity in both of the expressed arthropod insect kinin receptor assays (Nachman and Pietrantonio, unpublished data) and preliminary studies indicate it demonstrates statistically significant activity in in vitro Malpighian tubule secretion assays of the mosquito *Aedes aegypti* and cricket *Acheta domesticus* at μM concentrations (Nachman and Coast, unpublished data). The completion of full dose-response curves for the in vitro diuretic assays and an evaluation of in vivo biological activity await future experiments. Future evaluations of this and other rationally designed, indole-containing libraries may identify other nonpeptide mimetic agonists as well as antagonists. The data further underscores the preeminent importance of the indole moiety of Trp^4 to the interaction of the insect kinins with their receptors.

Prospects for Enhanced Topical and/or Oral Bioavailability

Insect neuropeptides in general are not suitably designed to efficiently penetrate either the outer cuticle or the digestive tract of insects. Nonetheless, studies have shown that at least one class of insect neuropeptides can be modified to enhance bioavailability characteristics.

Topical Activity

Topical experiments have not been conducted with the insect kinins, but have been conducted with a couple of other insect neuropeptide families. Topical application of members of the adipokinetic hormone (AKH) family in mixed aqueous/organic solvent to the cuticle of the cricket *Gryllus bimaculatus* did lead to a significant AKH-like increase in hemolymph lipids.[64] The AKH family as a group of peptides are particularly hydrophobic, a factor that may aid their penetration through the hydrophobic cuticular waxes. On the other hand, experiments involving topical application of aqueous solutions of members of the pyrokinin/PBAN family did not produce significant pheromone production in the tobacco budworm moth *Heliothis virescens*.[65,66] Structural modification to produce pyrokinin analogs that feature amphiphilic properties greatly enhances their ability to both penetrate the hydrophobic cuticle and also to maintain the aqueous solubility required to reach their target receptor once they encounter the hemolymph.[65,66] The pyrokinin/PBAN family shares a common C-terminal pentapeptide FXPRLa and regulates a number of physiological processes, including regulation of sex pheromone biosynthesis in females of Lepidoptera.[67-69] The development of a series of pseudopeptide analogs of this neuropeptide family began with the addition of various hydrophobic groups to the N-terminus of the C-terminal pentapeptide active core, which in conjunction with the polar/charged Arg side chain, confer an amphiphilic property. Hydrophobic groups appended to the N-terminus included fatty acids of various chain lengths, cholic acid, carboranylpropionic acid and aromatic acids.[46,65,70-73] Many of these amphiphilic analogs showed greater in vivo potency in a pheromonotropic assay than the native 33-membered pyrokinin PBAN when delivered via injection in female *H. virescens* moths. In studies involving topical application, neither PBAN nor its C-terminal pentapeptide active core elicited pheromone production when applied at 1-2 nmoles/female. By contrast, amphiphilic analogs induced significant pheromone production 15 min after topical application of aqueous solutions to the lateral abdominal surface of the moths with ED_{50} values ranging from 60 to 500 pmoles per female and ED_{max} values of 60-2000 pmoles per female.[71] It is worth emphasizing that this result was achieved without the assistance of organic solvent mixtures. When applied to dissected pieces of *H. virescens* cuticle, 24-hour recoveries of a series of amphiphilic pyrokinin analogs ranged from 5-70%. In addition, prolonged pheromone production exceeding 20 hours following a single topical application of an amphiphilic pyrokinin analog to *H. virescens* moths was observed. In addition, the nature of the hydrophobic moiety was observed to influence the duration of the slow release of a given amphiphilic pyrokinin analog. The results demonstrated that the insect cuticle could serve as a reservoir for the time-release of a physiologically active, amphiphilic analog of an insect neuropeptide.[71] The development of topically-active, amphiphilic analogs of the insect kinins would be a logical extension of these studies.

One amphiphilic pyrokinin analog, **2Abf-Suc**-FTPRLa, featured an appended brominated fluorine aromatic ring as the hydrophobic moiety and demonstrated highly unusual in vivo activity

following delivery via injection.[73] Unlike other amphiphilic analogs, a single injection of 500 pmoles of this brominated fluorine (2Abf) pyrokinin analog into female *H. virescens* moths induced a highly unnatural response; continuous production of high levels of pheromone for as long as 20 hours.[73] While such a result might be expected from the time-release of an amphiphilic analog following topical application, the observed prolonged pheromone production following injection suggested that the 2Abf analog might have a strong affinity for and/or interaction with, the pheromone receptor. Indeed, recent studies on an expressed pyrokinin/PBAN receptor from *H. virescens*, the Abf analog proved to be more active than the native 33-membered PBAN neuropeptide and considerably more active than the parent C-terminal pentapeptide fragment (Nachman and Adams, unpublished data). However, the analog had an interesting side effect. That is, it induced mortality in 100% of the treated moths. The LC_{50} value for this potent toxic side effect was found to be 0.7 pmoles and 100% mortality could be achieved with a 5 pmole dose. Related analogs such as **7Abf-Suc**-AARAAa and another pyrokinin analog that featured only the fluorine ring, both of which retained similar amphilphilic and solubility properties, did not demonstrate any toxicity.[73] Therefore, the toxicity was not a result of the presence of the 2Abf moiety. Furthermore, the toxic effect was highly specific to the presence of the pyrokinin sequence. Although the mechanism of the insecticidal activity of the 2Abf analog in *H. virescens* is not known, it is hypothesized that the specific nature of the toxicity results from an interaction of receptor sites for the pyrokinin/PBAN class of insect neuropeptides.[73] This class of neuropeptides has a wide range of known biological activities in insects in addition to pheromonotropic activity, including hindgut and oviduct myotropic activity, pupariation, induction of egg diapause, diapause break and melanotropic activity.

Oral Activity

No oral activity data has been reported for the insect kinin class of neuropeptides. Generally, oral activity for unmodified insect neuropeptides is poor to nonexistent. Small quantities of members of the pyrokinin/PBAN[74] and the proctolin classes of neuropeptides[75] have been reported to survive exposure to the digestive enzymes and pH of the digestive tract and penetrate through to the hemolymph to reach their target receptors. In addition, small quantities (<3%) of A-type allatostatins have been shown to be transported across dissected foregut tissue of the moth *Manduca sexta*.[76] An early attempt to feed the pyrokinin PBAN to adult females of the moth *H. zea* reported very low and inconsistent levels of pheromone production that were not progressively dose-dependent.[74] In other experiments, no statistically significant pheromone production was observed in starved adult females of the related moth species *H. virescens* 1-2 hours after ingestion of a sugar solution of 50 pmoles/μL of PBAN or the C-terminal pentapeptide core FTPRLa. However, biostable amphiphilic, pyrokinin analogs **Hex**-FT**(Hyp)**RLa (901) and **Hex**-FT**(Oic)**RLa (904) demonstrated an ability to penetrate the dissected portions of the insect digestive tract as well as significant oral activity.[3] The components hydroxyproline (Hyp) and octahydroindole-2-carboxylate (Oic) were incorporated as sterically-hindered replacements for Pro to enhance resistance to tissue-bound peptidases that would be encountered in hemolymph. Indeed, these analogs proved to be completely resistant to degradation by peptidase bound to Heliothine Malpighian tubule tissue over a 120 min period, whereas a natural pyrokinin was completely degraded in 30 min. Direct penetration of the two analogs through dissected cockroach foregut and midgut were investigated. The digestive system of the cockroach was chosen because the guts of adult moths are not of sufficient size or stability to allow for practical delivery of peptide analog solutions. Indeed, Figure 9 shows that out of a total of 2.5 nmoles placed within the lumen of a sealed foregut, 800 nmoles (over 30%) of Oic analog 904 penetrated the tissue preparation. It is interesting to note that Oic analog 904 demonstrates time-release properties, as equal amounts were recovered over the 0-4 hour period as over the 4-24 hour period. The majority of Hyp analog 901 penetrated in the first 0-4 hour period.[46] The lumen of the insect foregut features a cuticular component, which could explain why the time-release effect is similar to that observed for the outer cuticle for these amphiphilic analogs. It also suggests that the foregut can serve as a reservoir for the time-release delivery of neuropeptide analogs in insects, thereby bypassing the hostile, peptidase-rich environment of the midgut.

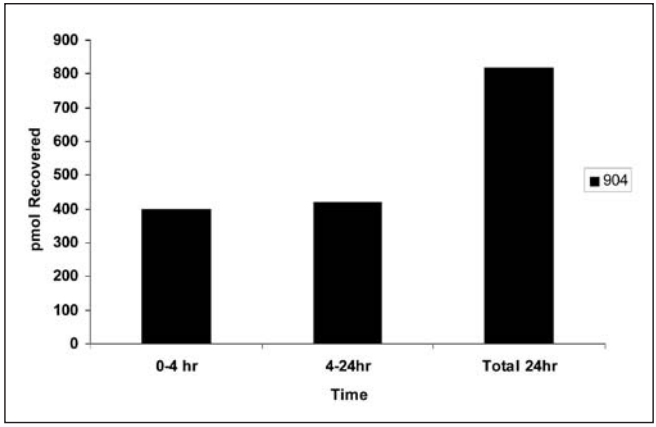

Figure 9. Amount of Oic-pyrokinin analog 904 penetrating isolated, ligated cockroach foregut preparations over indicated time periods from an initial 2.5 nmoles. From Nachman et al (2002b).[46]

These in vitro penetration studies of analogs 901 and 904 were followed by in vivo oral pheromonotropic activity trials in adult female *H. virescens*.[46] Pheromone production was monitored following ingestion of 30 μL of a sugar solution containing 50 pmoles/μL of either 901 or 904 at 1.5, 3, 4 and 6 hours post feeding (Fig. 10). A statistically significant increase in pheromone titer was observed at 1.5 hours postfeed with 901 with a 17% maximal response. Oral administration of the analog 904 induced statistically significant levels of pheromone at 1.5, 3 and 4 hours postfeed, but not at 6 hours. Optimal pheromone production was achieved at 3 hours, with a highly significant ~60% maximal response.[46] The shift in the pheromone spike from 1.5 hours for 901 to 3 hours post-feed for 904 is consistent with the greater time-release effect observed for the direct penetration of the more hydrophobic 904 in both ligated fore-and midgut preparations.

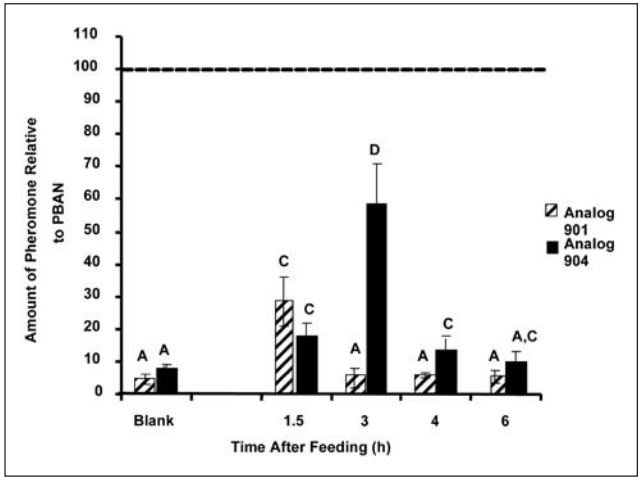

Figure 10. Amount of pheromone, relative to maximal levels produced by injected PBAN, produced by Hyp-pyrokinin analog 901 and Oic-pyrokinin analog 904, at 1.5, 3, 4 and 6 hours following oral administration. The dotted line at 100% denotes maximal production of pheromone by injected PBAN (positive control). From Nachman et al (2002b).[46]

The development of orally-active, biostable amphiphilic analogs of the insect kinins would be a logical extension of these studies.

Conclusion

Evaluation of truncated analogs, an Ala-replacement series and restricted conformation analogs incorporating turn-mimic components on recombinant arthropod expressed receptors for the insect kinins and biological assays in arthropods provided a structure/conformation-activity profile for this important class of neuropeptides. The insect kinins require a C-terminal pentapeptide as the minimal core sequence for activity, although full activity in expressed mosquito receptors and in housefly Malpighian tubule assays requires at least a C-terminal hexapeptide fragment. Replacement of residues Phe^1 and Trp^4 with Ala led to peptide analogs devoid of agonist activity and are, therefore, the most critical for an agonist response. However, while the analog in which Ala replaces Phe^1 is completely inactive, the analog in which Ala replaces Trp^4 demonstrates antagonism in in vitro cricket diuretic assays. Furthermore, a novel insect kinin analog featuring a C-terminal aldehyde group in which Phe^1 is replaced with an Ala retains weak activity in in vitro cricket diuretic assays. These results suggest that the Trp^4 is the most important for receptor binding. The evidence further suggests that the insect kinins adopt a *cis*Pro Type VI turn over residues 1-4, allowing the side chains of Phe^1 and Trp^4 to form an optimal aromatic surface during successful receptor interaction. A rationally-designed nonpeptide recombinant library based on the 'indole' side chain functional group of $Trp,^4$ the most critical of amino acids in the insect kinin core region, was synthesized and evaluated in expressed insect kinin receptors from the tick *Boophilus microplus* and mosquito *Aedes aegypti*. An insect kinin mimetic analog from this imidazopyridoindole library demonstrated a significant interaction with both expressed receptors as well as retention of fluid secretion activity in Malpighian tubules of the mosquito and cricket. Further evaluation of this and other rationally designed nonpeptide libraries can potentially yield other biostable agonists and antagonists of the insect kinins. Primary and secondary tissue-bound peptidase hydrolysis sites have been identified in the C-terminal region and analogs containing either β-amino acids or α-aminoisobutyric acid (Aib) adjacent to these sites retain significant bioactivity and demonstrate greatly enhanced biostability. Two biostable analogs show a selective agonist response in the expressed tick receptor assay over the expressed mosquito receptor.

Another class of insect kinin analogs containing a C-terminal aldehyde demonstrates unusual properties. One C-terminal aldehyde analog enhances the weight-gain inhibition activity of the insect kinins in *H. zea* larvae to such an extent that it results in increased mortality. Another 'magic bullet' C-terminal aldehyde analog selectively targets housefly Malpighian tubules, the major organ of diuresis in insects and leads to marked inhibition of urine release. An agent capable of selective depression of fluid secretion would be expected to allow pesticides to achieve higher concentrations in the hemolymph; and, in turn, likely reduce the amount of toxin required to kill an insect.

While neuropeptides are not generally designed for penetration of the outside cuticle or the gut wall in large quantities, enhancement of bioavailability has been demonstrated in at least one other class of insect neuropeptides. Amphiphilic analogs of the pyrokinin/PBAN family of insect neuropeptides have shown an ability to efficiently penetrate in vitro preparations of insect cuticle and foregut, as well as demonstrate potent activity in in vivo pheromonotopic bioassays when administered via topical or oral routes. Amphiphilic analogs contain both polar and apolar components that confer surfactant-like characteristics. Similar modification of the insect kinins to impart amphiphilic character would present a likely path to the development of mimetic analogs with enhanced bioavailability.

In conclusion, the studies presented here have led to the identification of interesting tools for arthropod endocrinologists and promising mimetic analog leads in the development of selective, environmentally friendly arthropod pest control agents capable of disrupting insect kinin regulated processes.

Acknowledgements

We wish to acknowledge financial assistance from a grant from the USDA/DOD DWFP Initiative (#0500-32000-001-01R) (RJN), the North Atlantic Treaty Organization (NATO) Collaborative Research Grant (#LST.CLG.979226) (RJN), a NRI/USDA grant number 2003-35302-13678 (PVP) and a Texas Advanced Technology/Research Grant (#000517-0103-2001) (RJN).

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